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Mutations Seen Among Patients With Pheochromocytoma and Paraganglioma at a Referral Center From India

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Key words

- pheochromocytoma
- paraganglioma
- India
- SDHx
- genetic screening

Abstract

Determining the mutational status of susceptibility genes including *RET*, *VHL*, *SDHx* (*SDHB*, *SDHC*, *SDHD*) among patients with pheochromocytoma/paraganglioma (PCC/PGL) is gaining importance. These genes have not been systematically characterized among patients with PCC/PGL from India. The aim of the work was to screen the most frequently mutated genes among patients with PCC/PGL to determine the frequency and spectrum of mutations seen in this region. Fifty patients with PCC/PGL treated at our tertiary care hospital between January 2010 and June 2012 were screened for mutations in susceptibil-

ity genes using an algorithmic approach. Thirty-two percent (16/50) of patients were found to be positive for mutations including mutations among *RET* (n=4), *VHL* (n=6), *SDHB* (n=3), and *SDHD* (n=3) genes. None of these patients were positive for *SDHC* mutations. A significant association was found between young patients with bilateral tumors and *VHL* mutations (p=0.002). Two of the 3 patients with extra-adrenal *SDHB* associated tumors, had unique mutations, viz., c.436delT (exon 5) and c.788_857del (exon 8), one of which was malignant. High frequency of mutations seen among patients in this study emphasizes the need to consider mutational analysis among Indian patients with PCC/PGL.

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare highly vascular neuroendocrine tumors [1,2]. Pheochromocytomas arise from chromaffin cells of the adrenal medulla and secrete catecholamines; approximately 10% of these tumors can arise from the extra adrenal chromaffin tissue and these are referred to as PGLs. PCC/PGLs are known to cause severe morbidity, attributed to the high burden of circulating catecholamines, and, in severe cases, can also result in death.

The understanding of genetic determinants of these tumors has been gradually evolving since early 2000s and a total of 12 genes are now known to be associated with hereditary PCC [3]. While 3 of these genes – *VHL* (von Hippel-Lindau), *NF1* (neurofibromatosis), and *RET* (multiple endocrine neoplasia type 2, MEN2) – are susceptibility genes associated with syndromic disease, germline mutations involving the succinate dehydrogenase (SDH) complex subunit genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*) and one of the cofactors of the complex (*SDHFA2*) are also

known to be associated with PCC/PGLs. In addition, evidence for the association of transmembrane protein 127 (*TMEM127*), *MYC*-associated factor X (*MAX*), Kinesin family member 1B (*KIF1Bβ*), and EGL 9 homologue 1 (*EGLN1/PHD2*) with hereditary PCC/PGL is also mounting. Screening for mutations is important since identification of these mutations can be a pointer of the risk of malignancy (*SDHB* mutations) or an underlying syndromic disease (*VHL*, *NF1*, *RET*), and can indicate recurrence or the presence of tumors at other sites (*TMEM127* or *SDHx*) [4,5]. Further, identification of germline *SDHx* mutations could indicate increased susceptibility of these patients for multiple primary tumors emphasizing the need to screen for these mutations.

The percentage of tumors reported to carry germline mutations has also increased with the expanding list of susceptibility genes. Initial conservative estimates of 10% hereditary tumors have gradually been replaced by studies with higher mutation positivity among PCC/PGLs [3,5]. Neumann et al. in 2002 described the presence of mutations among 24% of the apparently

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sporadic cases of PCC/PGLs [6]. However, the prevailing body of literature on PCC/PGLs indicates varying prevalence of mutations among unselected cases ranging from as low as 5.6% as seen in a Swedish cohort to as high as 41% in a recent report by Fishbein et al. [3,7,8]. Unfortunately, there have not been many studies from Asia that have systematically screened PCC/PGLs for mutations, though a Korean report with limited genetic testing indicates 13.2% of PCC/PGLs do carry germline mutations [9]. The cost associated with screening such patients is fairly prohibitory and perhaps explains the lack of data from the region. However, the suggestion that an algorithmic approach [10–15] that does not require the testing of all known susceptibility genes for each individual patient, but uses a step-wise approach to screening leading to cost reduction in testing, has opened new vistas for testing, even in a limited resource setting like India.

Most studies from India have been limited to clinical and radiological finding in these patients and there is complete paucity of information with regard to genetic mutations. Therefore, consecutive cases of PCC/PGLs presenting over a 2½ year period at our tertiary care hospital were characterized for germline mutations using the algorithmic approach suggested by Benn et al. [10]. Our objective was to determine the frequency and spectrum of mutations in our hospital-based cohort using limited genetic testing to deduce mutations in the frequently mutated genes associated with PCC/PGLs. To the best of our knowledge, this is the first report of germline mutational status among patients with PCC/PGL of Indian origin.

Patients and Methods

Patients

Our institution is a large tertiary care referral hospital, receiving patients from all over India. All consecutive cases of PCC/PGLs presenting between Jan 2010 and June 2012 were included in this study. After approval from the institutional review board, a written consent for genetic testing was obtained from all patients. Fifty patients were recruited and screened for clinical features associated with VHL, MEN2, and NF1. These 50 patients also included 2 families with 3 patients each, one with a history of bilateral adrenal PCC [16] and the other with familial carotid body tumors [17]. One patient with multiple neurofibromata was clinically diagnosed to have neurofibromatosis (NF1) and genetic analysis was not performed.

Testing for susceptibility genes

Peripheral venous blood was collected from patients included in the study and 200 µl was used for extraction of genomic DNA using QIAamp DNA blood minikit (QIAGEN, Hilden Germany). The algorithm suggested by Benn et al. [10] was followed for genetic screening of these samples and this was carried out in a step-wise manner using 20 picomoles of sequencing primers that covered intron-exon boundaries for *RET*, *VHL*, *SDHB*, *SDHD*, and *SDHC* genes [18–20]. If a sample was positive for a mutation, then the rest of the genes in the algorithm were not tested and therefore, all susceptibility genes were not tested for all cases. The reactions were carried out in a final volume of 25 µl containing 1U of AmpliTaq Gold (Applied Biosystems, USA) and amplified in a Veriti thermal cycler (Applied Biosystems, USA). The PCR products were detected using a 2% agarose gel and both the sense and antisense strands for all exons characterized were

sequenced using the ABI PRISM 3130 genetic analyzer with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The sequences were compared to wild type sequences. Large deletions for *VHL* were screened as described previously [21] using a SYBR Green based real-time quantitative PCR (RQ-PCR) and normalized using 2 reference genes with a normal copy number, that is, *ZNF80* (3q13.31) and *GPR15* (3q12.1). Deletions in the *SDHB* gene were screened using a fluorescent multiplex PCR gene dosage assay [22] that enables simultaneous amplification of multiple exonic fragments under quantitative conditions. An additional fragment, from the (*ALB*) gene, was coamplified as a control in each PCR. Data were analyzed using the GeneMapper software version 4.0 (Applied Biosystems) and each reaction was validated with positive and negative references.

Immunohistochemical analysis using *SDHB* antibodies became available since late 2011 [23], but samples were not triaged, despite the cost benefits it would offer since mutational analysis was being performed for the first time at our center.

Statistical analysis

The descriptive statistics was reported using n (%) for categorical variables and mean ± SD for continuous variables. Association between age and mutational status was assessed using Fisher's exact test.

Results

The age of these patients ranged from 10–70 years (median 32.5 years). The duration of symptoms ranged from 15 days to 4 years (median 2 years). Interestingly, a 10-year-old child, son of a patient with PCC, also developed hypertension and was therefore screened early for PCC [11]. Most patients presented with headache, hypertension, abdominal-pain, and decreased vision (Table 1) while the classic symptoms of chest pain, sweating, and palpitation were uncommon. The smallest tumor measured 3×3 cm and the largest measured 15×11.4×9.2 cm. No correlation was seen between the size of the tumor and the biochemical profile of these patients. Two of the tumors that were > 11 cm in size were associated with malignancy. Three patients from a single family had bilateral carotid body tumors and one of them in addition had multiple intra-abdominal PGLs. Five patients

Table 1 Clinical characteristics of patients with PCC/PGL in the study.

Total No.	50
Gender (M/F)	28:22
Age at Diagnosis (years)	
Mean ± SD	35.0 ± 14.69
Median (IQR)	32.50 (22.0, 47.25)
Range	10–70
Common presenting symptoms	
Headache	9
Hypertension	8
Abdominal pain	6
Decreased vision	5
Location of tumor	
Adrenal, unilateral	32
Adrenal, bilateral	9
Extra adrenal	5
Head and neck PGL	3
Bladder	1

Table 2 Age, spectrum of mutations, and clinical phenotype of patients with PCC/PGL.

Gender	Age (Years)	Gene	Exon	Clinical phenotype	Mutation	Protein alteration
M	50	RET	11	HT crisis, B/L PCC	c.1901G>A	p. Cys634Tyr
M	44	RET	11	HT, B/L PCC, MTC, PTA	c.1901 T>C	p.Cys634Arg
M	46	RET	11	Rt ADR, MTC	c.1901T>C	p.Cys634Arg
F	53	RET	16	MTC, Rt ADR, GN	c. 2753T>C	p.Met918Thr
M	33	VHL	3	B/L PCC, DM	c. 499C>T	p.Arg167Trp
F	21	VHL	3	Rt ADR	c. 533T>A	p.p.leu178Gln
F	18	VHL	2	B/L PCC, PNET	c. IVS2 + 3A>G	?splice site
F	17	VHL	1	B/L PCC	c.245G>T	p.Arg82Leu
M	14	VHL	1	B/L PCC	c.245G>T	p.Arg82Leu
M	10	VHL	1	B/L PCC	c.245G>T	p.Arg82Leu
F	25	SDHB	7	Bladder PGL	Exon 7 deletion	p.?
M	35	SDHB	5	HT, DM, PGL	c.436delT	p.?
M	41	SDHB	8	PGL	c.788_857del	p.?
M	32	SDHD	4	PGL, B/L carotid body tumor	c.337_340delGACT	p.Asp113M
F	29	SDHD	4	PGL, B/L carotid body tumor	c.337_340delGACT	p.Asp113M
M	36	SDHD	4	PGL, B/L carotid body tumor	c.337_340delGACT	p.Asp113M

M: Male; F: Female; Rt: Right; Lt: Left; B/L: Bilateral; PGL: Paraganglioma; PCC: Pheochromocytoma; HT: Hypertension; DM: Diabetes mellitus; ADR: Adrenal; PNET: pancreatic neuroendocrine tumor; MTC: Medullary thyroid carcinoma; PTA: Parathyroid adenoma; GN: Ganglioneuroma

had metastatic tumors, one of which had spread to the omentum and one to the bone. Three patients with benign disease came back with recurrent tumors. The details of treatment for these patients are as follows: open surgery (n=24), laparoscopic surgery (n=20), carotid body excision (n=3), and partial cystectomy (n=1). One patient with extensive disease was treated with palliative chemotherapy and one refused surgery. Eight patients underwent surgeries at other centers before presenting to us.

The results of mutational analysis and the spectrum of mutations seen in this cohort of PCC/PGLs from India are shown in **Table 2**. Thirty-two percent (n=16) of all patients included in the study were positive for germline mutations in any one of the susceptibility genes being tested. These 16 included patients with *RET* (n=4), *VHL* (n=6), *SDHB* (n=3), and *SDHD* (n=3) mutations. None harbored *SDHC* mutations. While mutations seen in the *RET* [24] and *VHL* genes (www.lovd.nl) among the patients in this study have been described before, 2 unique mutations in the *SDHB* gene, viz. c.436delT (exon 5) and c.788_857del (exon 8), not previously described, were also seen. Both these novel variants were seen in patients with paraganglioma; the former also had metastatic disease. One case of a large deletion involving exon 7 of the *SDHB* gene was seen in a patient with a bladder tumor [17].

There were 9 patients with bilateral adrenal tumors, 7 (7/9) of whom were found to carry mutations in either the *RET* (n=2) or the *VHL* (n=5) genes (**Table 2**). A significant association was found between young patients (<22 years of age) with bilateral tumors and *VHL* mutations (p=0.002). However, the mutation positivity among patients with unilateral adrenal tumors was low with only one of the 32 cases carrying a mutation. All 3 patients with *SDHB* mutations had extra-adrenal tumors, though only one of them was malignant. Among the 5 malignant cases only one was positive for an *SDHB* mutation, while the other 4 were negative for all susceptibility genes tested by the algorithmic approach. None of the patients with benign recurrent tumors were positive for mutations.

Discussion



To the best of our knowledge, pheochromocytomas (PCCs) and paragangliomas (PGLs) in Indian patients have not been characterized for mutations in associated susceptibility genes resulting in paucity of information in this context. The data presented here helps to highlight the frequency and spectrum of mutations seen among PCC/PGL patients in our country. Of the 50 patients with PCC/PGL included in the study, 32% harbored a germline mutation. The frequency of mutations reported worldwide has varied ranging from a mere 5% to 41% [3,5], though most of these reports are from Europe and North America. There is very little information from Asia with a lone report from Korea showing a frequency of 13.2% [9]. Therefore, the frequency reported here is of significance since screening for susceptibility genes among PCC/PGLs is not a routine practice in this region and might help to overcome the lacunae in this area. As our hospital is a tertiary care referral center, receiving patients from all over the country, the frequency reported here could perhaps be extrapolated to the rest of the country.

Though the percentage of patients with germline mutations was fairly high in this study (32%), it is important to note that most patients were classified as affected with apparently sporadic PCC/PGL, until mutation analysis was performed. This affirms the fact that patients who might not present with a family history could still be at risk of carrying these mutations and will certainly need to be screened. However, it is important to note that genetic screening in this study was limited to 6 genes that are commonly found to be mutated among PCC/PGLs and mutations with *TMEM 127*, *MAX*, *SDHA*, *SDHAF2*, *KIF11B*, and *PHD2* were not screened. But, studies characterizing these mutations have attributed very few cases [25,26] to such mutations and we believe that our results might not have altered significantly even if these rare and uncommon mutations were screened among the patients in this study.

Mutations in the *VHL* gene were the most frequent (n=6) mutations seen in this study and were commoner among young patients with bilateral tumors (p=0.002). Other studies have also described a similar genotype-phenotype association [1,3,27,28]. One patient with a *VHL* mutation in this cohort also had a pancreatic neuroendocrine tumor. A close follow-up of

these cases with *VHL* mutations will help in the early detection of other tumors thereby reducing morbidity and mortality and will profoundly alter the management of these patients.

The frequency of mutations in *SDHD* [22, 29] and *SDHB* genes were similar ($n=3$ in each) and all *SDHB* positive tumors, as is commonly known, were extra-adrenal in location [1,3,15]. However, only one of the *SDHB* positive tumors was malignant. While the cases in our cohort would require longer follow-up, a similar study from Sweden has also shown that even after a mean follow-up of 23.3 years, none of the tumors with *SDHB* mutations turned malignant [7]. Also, both the *SDHB* positive cases in this cohort with no malignancy had deletions, one with a unique deletion (c.788_857del, exon 8) that has not been described at other centers and the other had an exon 8 large deletion. Though these mutations might not be the singular genetic change aiding in malignant transformation [24,30,31] it would nevertheless be interesting to characterize these mutations by classical and bioinformatic approaches to determine if these molecular changes do impact the activity of SDHB protein and its interaction in various cellular pathways.

Interestingly, only one of the 5 (20%) cases of malignant PCC/PGLs in this study were associated with *SDHB* mutations unlike other studies where ~50% were *SDHB* positive [5,7,32]. These mutation negative samples in patients with underlying malignancy are ideal samples for targeted next generation sequencing (NGS) to identify mutations in the newly described susceptibility genes [33]. Rattenberry et al. [33] have reported that NGS based testing in their set up was not only comprehensive but also less expensive than Sanger sequencing and therefore in the long run this technology might prove to be a boon to patients even in developing countries.

Early identification of these mutations is important in order to monitor tumor recurrence, malignancy, and the development of additional tumors. The abovementioned benefits of screening taken along with the high percentage of mutations in this study, is a pointer to consider routine screening of all patients with PCC/PGL. However, in a developing country where most people lack medical insurance the cost of screening could become the single most prohibitive factor. Triaging with antibodies and a step-wise screening approach would perhaps be more feasible at this point in time [6,23,34].

Our study has several limitations including the use of an algorithmic approach based on phenotypic and clinical findings, the small number of cases included in this cohort, not detecting all known mutations/minor mutations associated with PCC/PGLs and limited follow-up in some cases. Despite these drawbacks, this study highlights the frequency of mutations in India and focuses on the need to test for susceptibility genes even in a setting with limited resource. Finally, the data from this study will help in designing other prospective studies and can be of value in guiding genetic testing within the country.

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Conflict of Interest



The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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